NSPS, A POTD1 HOMOLOG, ACTS AS A SPERMIDINE SIGNAL SENSOR, NOT A TRANSPORTER, IN *VIBRIO CHOLERAE*

A Thesis
by
Rebecca Elizabeth Cooper

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APPROVED BY:

_____
Ece Karatan
Chairperson, Thesis Committee

_____
Sue L. Edwards
Member, Thesis Committee

_____
Annkatrin Rose
Member, Thesis Committee

_____
Steve W. Seagle
Chairperson, Department of Biology

_____
Edelma D. Huntley
Dean, Research and Graduate Studies

FOREWORD

The organization and formatting of this thesis strictly follows the instruction to the author for manuscript submission to *Research in Microbiology*, a publication of the Institut Pasteur. The general organization of the text is similar to that of a research paper, with the whole text in a four main sections headed “Introduction,” “Materials and Methods,” “Results,” and “Discussion.”

ABSTRACT

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(August 2010)

Rebecca Elizabeth Cooper, B.S., Georgia Institute of Technology

M.S., Appalachian State University

Chairperson: Ece Karatan

Biofilm formation is important for the survival of *Vibrio cholerae* in its natural aquatic environments and within the human intestinal tract. We have previously shown that the absence of PotD1, the periplasmic binding protein for the spermidine ABC transporter, leads to an increase in biofilm formation. In addition, spermidine reduces biofilm formation through its interaction with NspS, a homolog of PotD1. Due to its similarity to PotD1, NspS has been annotated as a polyamine transporter. In this study, we seek to establish whether NspS is capable of transporting spermidine into the cell. We show that the absence of NspS did not lead to a loss of spermidine inside the cell, indicating that NspS does not function as a transporter. Polyamine analysis of wild-type, Δ*nspS, ΔpotD1*, Δ*nspSΔpotD1*, and the Δ*nspSΔpotD2*Δ*potD1* strains suggested that there are no high affinity transporters present in the cell in addition to PotD1. Biofilm assays showed that the biofilm cell densities of Δ*nspSΔpotD1* mutant were intermediate between the *ΔnspS* and *ΔpotD1* single mutants, implicating neither NspS nor PotD1 are epistatic over the other. Our results suggest that NspS plays a role in spermidine signaling, but not transport in *V. cholerae.*

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1. Introduction

Vibrio cholerae is a Gram-negative curved-rod shaped bacterium with a polar flagellum. In the 19th century, *V. cholerae* was confirmed to be the causative agent of cholera disease by Robert Koch, although the bacterium had been implicated as the cause of cholera as early as the 1850's when John Snow correlated the disease with contaminated water [5, 12, 13, 15, 29]. Cholera disease is characterized by the colonization of the gastrointestinal tract by *V. cholerae,* with symptoms including severe diarrheal disease, dehydration, and decreased blood pressure. The bacterium is also a natural inhabitant of salt, brackish, and fresh water environments and is often found attached to copepods, phytoplankton, insects, and crustaceans [4, 7, 13, 21, 36]. The organism can be found in a free-living (planktonic) form or attached to a solid surface in its sessile state (biofilm) [3, 19, 36]. The capacity of *V. cholerae* to form biofilms in aquatic environments can lead to greater survival and increased rate of disease transmission [3, 8, 29].

Biofilms are composed of multiple layers of cells attached to a surface and surrounded by an extracellular matrix. Biofilms form as a survival mechanism, often times making the bacteria resistant to environmental challenges, such as pH changes, the presence of free radicals, disinfectants, natural antibiotics, and nutrient deprivation [6, 9, 18, 19, 25, 31]. In *V. cholerae*, *vps* (*Vibrio* polysaccharide) genes encode enzymes that are required for the synthesis of the exopolysaccharide, VPS [2, 11]. The formation of mature biofilm structures is dependent on the presence of the *vps* genes [2, 27].

Biofilm formation by *V. cholerae* is regulated by various environmental signals that cue the bacteria to transform from their planktonic form into a sessile mode. Environmental signals that are linked to biofilm formation in *V. cholerae* include mechanical signals, nutritional and metabolic signals, such as the presence of glucose, indole, polyamines, and catabolite repression, inorganic molecules, such as iron and phosphate, osmolarity, antimicrobial compounds, host-derived signals, such as bile acids, and quorum sensing signals [17, 18]. Polyamines are one of the recently identified environmental signals known to influence biofilm formation in *V. cholerae*. Polyamines are small aliphatic hydrocarbon molecules with quaternary nitrogen groups that possess a net positive charge at physiological pH (Fig. 1) [34]. Polyamines are important for many functions in prokaryotes and eukaryotes, including RNA and protein synthesis, protection against oxidative damage, cell growth, and biofilm formation [1, 21, 22, 30, 33, 35]. In *V. cholerae*, putrescine and norspermidine are the most abundant polyamines present [20]. However, when grown in Luria-Bertani broth media, spermidine is also present in large amounts inside the cell as the result of import from the external environment [20, 23]. *V. cholerae* has the capacity to synthesize putrescine, cadaverine, diaminopropane, and norspermidine; the bacteria lack the ability to synthesize spermidine in large amounts. However, *V. cholerae* can import exogenous spermidine into the cell through a spermidine transporter [23].

Polyamine transport is facilitated by specific transporter systems in bacteria. Polyamine transport systems have the capacity to uptake exogenous polyamines in the event that polyamine biosynthesis is interrupted or non-existent. In the model organism *Escherichia coli*, it has been shown that spermidine and putrescine are transported by ATP-binding cassette (ABC) transporters encoded by two different polyamine transport (Pot)

operons. The *potABCD* operon codes for the transport system that is responsible for the preferential uptake of spermidine, while the *potFGHI* operon codes for the transport system that is responsible for putrescine uptake [14].

In *V. cholerae*, a polyamine transport operon, *potABCD2D1*, homologous to the transport system found in *E. coli* has been identified [23]. Previous work has shown that *V. cholerae* PotD1 protein is responsible for spermidine transport as spermidine is absent inside the cell when the *potD1* gene is deleted [23]. The exact role of PotD2 has yet to be fully established, but it is possible that the protein functions in the transport of other, yet unidentified polyamines. *V. cholerae* polyamine transport systems have been implicated in the regulation of biofilm formation. The absence of PotD1 leads to an increase in biofilm formation, whereas the deletion of PotD2 has no effect. These results suggest that PotD1 represses biofilm formation, directly or indirectly, as a result of spermidine import into the cell. In addition, the PotD1 paralog, NspS, has also been shown to regulate biofilm formation in response to exogenous spermidine and norspermidine [16, 23].

NspS has been previously annotated as a spermidine transporter protein, based on sequence similarity to the *E. coli* PotD protein sequence and the *V. cholerae* PotD1 protein sequence. However, the role of NspS in polyamine transport has not been studied. Furthermore, earlier studies suggest that NspS functions in the transduction of polyamine signals, rather than their transport [16]. In this study, our objective was to further characterize spermidine transport in *V. cholerae* and its role in regulating biofilm formation. We wanted to establish whether NspS is capable of transporting spermidine into the cell, whether there are any other high affinity or low affinity spermidine transporters, and finally

whether there is an epistasis relationship between NspS and PotD1 with respect to their effect on biofilm formation.

2. Materials and Methods

2.1. Bacterial strains, plasmids, media, and reagents

The bacterial strains and plasmids used in this study are listed in Table 1. For all experiments, *V. cholerae* serotype O139 strain MO10 was used. All experiments were conducted with Luria-Bertani (LB) agar or broth. When required, the growth media were supplemented with antibiotics at the following concentrations: ampicillin, 100 µg/ml; streptomycin, 100 µg/ml; carbenicillin, 50 µg/ml. Plasmids were prepared using a Promega Wizard Plus SV Minipreps DNA Purification System (Madison, WI). Plasmids were introduced into *E. coli* and *V. cholerae* strains via electroporation using a Bio-Rad MicroPulser (Hercules, CA). Norspermidine, spermidine, diaminopropane, cadaverine, diaminooctane, benzoyl chloride, trichloroacetic acid, and HPLC-grade methanol were purchased from Sigma (St. Louis, MO). Putrescine was obtained from Research Organics (Cleveland, OH). Phire Hot Start DNA polymerase was purchased from New England Biolabs (Ipswich, MA).

2.2. Construction of ΔnspSΔpotD1 and ΔnspSΔpotD1ΔpotD2 mutants

The *ΔnspSΔpotD1* and *ΔnspSΔpotD1ΔpotD2* deletion mutants were created via double homologous recombination with sucrose selection as described previously [24]. Briefly, to make the *ΔnspSΔpotD1* or the *ΔnspSΔpotD1ΔpotD2* mutants, AK058 or AK082 were mated with PW514, respectively. The bacteria were next plated on selection

agar (LB media supplemented with 100 µg/ml streptomycin and 50 µg/ml ampicillin) and the antibiotic resistant colonies were purified by restreaking on selection agar to confirm single crossover events. Next, colonies were first streaked on non-selective media followed by incubation on agar plates containing 30% sucrose plates [24]. Sucrose resistant colonies were screened for recombination to the mutant genotype using PCR.

2.3. Biofilm formation assay and growth analysis

Biofilm assays were performed to determine the phenotypes of the *ΔnspS, ΔpotD1, ΔpotD1ΔpotD2, ΔnspSΔpotD1, ΔnspSΔpotD1ΔpotD2* deletion mutants. Briefly, 300 µL of LB growth medium was added to borosilicate tubes. The growth medium was supplemented with 1 mM spermidine (Sigma) when needed. The tubes were inoculated with either the wild-type or the mutant strain of interest at an OD_{595} reading of 0.01. The cultures were incubated at 27°C for 24 hours. Planktonic cells were removed from the tubes; the remaining biofilm was washed twice with 300 µl of LB growth medium. The biofilm was disbanded by vortexing for 1 minute in the presence of 1-mm-diameter borosilicate glass beads (Biospec, Bartlesville, OK). The final planktonic cell densities and final biofilm cell densities were measured using 150 µl of each aforesaid cell suspension using a Bio-Rad MicroPlate Reader Model 680 (Hercules, CA). All assays were performed in duplicate and repeated three times. The data was pooled for analysis. Unpaired, 2-tailed t-tests were conducted on data collected from the biofilm assays using Microsoft Excel. The p-values calculated from each t-test were used to establish statistical significance, where a p-value less than 0.05 was deemed significant.

2.4. Polyamine extraction protocol

Polyamine extraction from the wild-type and mutant *V. cholerae* strains was essentially performed as previously described [23, 28, 32]. Briefly, 20 ml of cells were grown to mid-log phase at 27°C, pelleted, washed with Phosphate Buffered Saline (PBS) and resuspended in 10 µL of water per mg wet weight. Fifty nanomoles of diaminooctane was added to the cells as an internal standard and the cells were lysed by sonication. Cell debris was pelleted by centrifugation. Proteins were precipitated by the addition of 50% trichloroacetic acid and removed by centrifugation at 16000 rpm for 1 minute. The supernatant containing the polyamines was removed and used for benzoylation.

2.5. Polyamine benzoylation

Polyamine benzoylation of the cell extract and standard mix solution was performed to determine the polyamines present in the wild-type and mutant strains. The standard mix solution contained 50 nanomoles each of norspermidine, spermidine, putrescine, cadaverine, diaminopropane, and diaminooctane. Briefly, 2 mL of 2 M (8.0 g/100 mL) NaOH was added to 500 µL of each polyamine mixture or cell extract and vortexed. Then, 50 µL of 25% benzoyl chloride (in HPLC-grade methanol) was added. The mixture was vortexed for 1 minute and placed in the shaker incubator at room temperature for 1 hour at 150 rpm to allow for the benzoylation reaction to proceed. Following the incubation period, the reaction was extracted twice with 1 mL of chloroform. The chloroform was allowed to evaporate and the dry residue of benzoylated polyamines was dissolved in 100 µL of mobile phase (60% HPLC-grade methanol in 40% HPLC-grade water). The benzoylated product was used in the HPLC analysis to assess polyamine content in the wild-type and mutant cells.

2.6. Detection of polyamines via HPLC analysis

Benzoylated polyamines were analyzed using a Waters Breeze HPLC system with a 20 μL sample loop (Waters Corporation, Milford MA). Specifically, a Waters 1525 Binary Pump with a 2487 Dual Wavelength Absorbance Detector and a Waters Spherisorb ODS2 column (5μm, 250 x 4.6mm), fitted with a 50x4.6mm guard cartridge (Waters Corporation, Milford MA) was used as a stationary phase and a 60% methanol/40% water mobile phase was used for separation of the products. The HPLC run was performed isocratically, using the aforementioned 60% methanol/40% water solution as the mobile phase at a flow rate of 0.8 ml/min for 30 minutes. The detector was set at 254 nm. For peak integration Waters Breeze 2 software was used (Waters Corporation, Milford MA). Polyamine content was quantified by comparison to the standards; the internal standard diaminooctane (DAO) was used to correct for sample to sample variations. Each experiment was performed a minimum of three times to confirm reproducibility. Data from one experiment representative of the results are included.

3. Results

3.1. NspS is not a spermidine transporter.

In order to establish whether or not NspS is capable of transporting spermidine into the cell, we quantified the polyamines in the wild-type and the Δ*nspS* mutant. Wild-type *V. cholerae* grown in LB media contained putrescine, diaminopropane, cadaverine, norspermidine, and spermidine as expected (Fig. 1B). Because *V. cholerae* is not capable of producing significant amounts of spermidine endogenously under these conditions, the presence of intracellular spermidine is attributed to the import of spermidine found in the LB media (approximately 40 μM) [23]. The polyamine content in the Δ*nspS* mutant was similar to that of the wild-type. Presence of spermidine in the Δ*nspS* mutant shows that this mutant still retains the ability to transport spermidine. This result indicates that NspS is not functioning as a spermidine transporter.

3.2. PotD1 is the only high affinity spermidine transporter in V. cholerae.

PotD1 is necessary for the import of spermidine into *V. cholerae* [23]. To test for the presence of any other spermidine transporters in the *V. cholerae* genome, a double mutant lacking the *nspS* and *potD1* genes (Δ*nspSΔpotD1)* and a triple mutant lacking the *nspS*, *potD1*, and *potD2* genes (Δ*nspSΔpotD1ΔpotD2)* were constructed. Δ*potD1* mutant contained all the polyamines present in the wild-type, except spermidine, as expected (Fig. 2B). The polyamine contents of the Δ*nspSΔpotD1* and Δ*nspSΔpotD1*Δ*potD2* mutants were similar to

that of the *ΔpotD1* mutant, indicating that these strains also lack the ability to import spermidine (Fig. 2C and D). These data show that PotD1 is the only protein involved in spermidine transport under the conditions of our experiments. Furthermore, the lack of spermidine in the cell extract of the Δ*nspSΔpotD1*Δ*potD2* mutant indicates that there are no other high affinity transporters present.

3.3. Assessment of the presence of low affinity transporters in the V. cholerae genome.

The experiments described above indicate that there are no other high affinity spermidine transporters that are active in *V. cholerae* under the conditions of our experiment. These experiments were conducted in LB broth which has approximately 40 μM spermidine, thus do not rule out the possibility that low affinity spermidine transporters could still be present [23]. We evaluated this possibility by adding high concentrations of spermidine (1 mM spermidine) to the growth medium and analyzing the polyamine content of the cells. Spermidine was present in the cell extracts of the wild-type and the Δ*nspS* mutant at slightly elevated levels, indicating that NspS is not likely to be involved in spermidine transport even if spermidine is present at high concentrations in the environment (Fig. 3A-B). Interestingly, spermidine was present in the cell extracts of *ΔpotD1*, Δ*nspSΔpotD1,* and Δ*nspSΔpotD1ΔpotD2* mutants as well (Fig. 3C-E). Presence of significant amounts of spermidine in the triple mutant suggested that an alternative low affinity transporter may be present in the genome. An alternative explanation for the presence of spermidine in the cell extracts of mutants lacking the *potD1* gene is the possibility that the positively charged spermidine is attracted to the negatively charged polysaccharides that comprise the outer membrane O-antigen. This interaction could result in the extracellular spermidine mixing in

with the cell extract upon sonication of the cells and the presence of spermidine in the cell extract could be primarily an artifact of the experimental procedures. To differentiate between these possibilities, we modified our experimental procedure as follows: spermidine was added to the cells at the end of the incubation period, the cells were incubated at 4°C for 10 minutes before being pelleted. We hypothesized that the short incubation time and the low temperature would slow down the transport process such that spermidine accumulation in the cell would be minimal. We still observed significant amounts of spermidine in the cell even in the absence of PotD1 (Fig. 4A-C). We then modified the procedure again where we added the spermidine to cells immediately prior to centrifugation. We hypothesized that minimizing the amount of time cells came into contact with spermidine would ensure that no transport would take place, thus any spermidine we observed in the cell extract would likely be due to the experimental procedure. Again, spermidine was present in the cell extracts even in the absence of PotD1 (Fig. 4D-F).

3.4. Identification of VCA1113 as a possible low affinity transporter.

Experiments described above using the modified polyamine extraction protocol suggested that presence of spermidine in the cell extracts is likely to be an experimental artifact. Nevertheless, we wanted to determine if there are other potential candidates for low affinity spermidine transport in the *V. cholerae* genome. BLAST searches in the *V. cholerae* genome using the *E. coli* spermidine transport protein, PotD, yielded several homologs, including PotD1, PotD2, NspS, and also a putative transporter found on the 2nd chromosome at locus VCA1113. Protein sequence analyses indicated that there is a 43% identity between VCA1113 and PotD1; however, only a 23% identity exists between NspS and PotD1 (Fig. 5).

It is possible that this protein has a high affinity for an unknown polyamine, yet can still function as a spermidine transporter when spermidine is present in high concentration in the external environment, thereby implicating a role for VCA1113 protein as a low affinity spermidine transporter.

3.5. Analysis of dominant effects of the signaling mechanism of NspS versus the transporting mechanism of PotD1 on biofilm formation in V. cholerae.

Previous studies have shown that it is likely that NspS functions as a signaling protein [16] and PotD1 functions as a spermidine transporter protein [23], and both proteins have been shown to be involved in biofilm formation. NspS and PotD1 have opposite effects on biofilm formation, such that NspS is a positive regulator of biofilm formation while PotD1 functions as a negative regulator of biofilm formation. We wanted to understand if there is an epistatic relationship between NspS or PotD1 with respect to their regulation of biofilm formation in the presence and absence of environmental spermidine. Biofilm assays were conducted using the wild-type *V. cholerae* strain as well as the single (Δ*nspS, ΔpotD1)*, double (Δ*nspSΔpotD1)*, and triple (Δ*nspSΔpotD1ΔpotD2)* mutants in the absence of exogenous polyamines and in the presence of 1 mM spermidine (Fig. 6). The Δ*nspS* and the Δ*potD1* mutant strains exhibited significantly decreased and increased biofilm formation, respectively, as has been previously reported. However, the Δ*nspSΔpotD1* double mutant strain and the Δ*nspSΔpotD1ΔpotD2* triple mutant strain yielded a biofilm cell density intermediate to that of the Δ*nspS* and Δ*potD1*single mutant biofilm cell densities. This result suggests that NspS and PotD1 do not have an epistatic relationship; rather the NspS and PotD1 pathways provide separate inputs to regulate biofilm formation. Upon adding high

concentrations of spermidine (1 mM) to the growth medium, a decrease in biofilm formed by the wild-type and *ΔpotD1* mutant was observed; however, spermidine had no effect on biofilm formation in the mutants lacking the *nspS* gene (Δ*nspS*, Δ*nspSΔpotD1,* Δ*nspSΔpotD1ΔpotD2*). These results indicate there is a spermidine-induced decrease in biofilm formation in *V. cholerae* that is regulated via *nspS*, a finding that confirms previously reported results. Finally, the biofilm cell densities of the Δ*nspSΔpotD1* double mutant were similar to that of the wild-type strain whereas the biofilm cell densities of the Δ*nspSΔpotD1*Δ*potD2* triple mutant were significantly reduced ($P=8.93x10^{-5}$). This result suggests that in the absence of both *nspS* and *potD1,* PotD2 may have a stimulatory effect on biofilm formation.

4. Discussion

In this work, our goal was to investigate the relationship between polyamines and biofilm formation in *V. cholerae*. The link between biofilm formation and polyamine transport and polyamine signaling has not been well established, thus our intention was to evaluate the role polyamines play in the regulation of biofilm formation in *V. cholerae*. Additionally, we aimed to establish whether or not NspS responds to high concentration of spermidine as the result of a signaling event, a transporting event, or whether or not signaling and transport coalesce to regulate biofilm formation in *V. cholerae.*

In general, similarity in gene sequence correlates to similarity in function. Similarity between the transport genes found in *V. cholerae* and the transport genes found in other bacteria implicate a similarity in function. The NspS protein is a paralog of PotD1, with a 23% identity between the two protein sequences. Despite the sequence similarity between NspS and PotD1, data collected in this study suggests that NspS does not function as a spermidine transporter; our data illustrates that spermidine transport is unaffected in the absence of NspS. Specifically, the data indicates the effect of spermidine on NspS, and ultimately biofilm formation, is through a signaling mechanism. By establishing the function of NspS as a spermidine signal sensor, we negated the possibility of NspS functioning as a transporter protein.

This data supports previous studies that suggest PotD1 functions as a spermidine transporter, and expands the previous findings by establishing that there are no other high affinity transporters present in *V. cholerae*. Furthermore, the data provides evidence for the

possibility of a low affinity transporter being present in *V. cholerae*. In the presence of high concentration of spermidine (1 mM), polyamine content analyses showed the presence of spermidine in the cell extract in mutants lacking the *potD1* gene. We have identified VCA1113 as a proposed low affinity spermidine transporter based on BLAST searches involving homologs of PotD proteins. It is likely that VCA1113 functions as a high affinity transporter for an unknown polyamine, but has the capacity to function as a low affinity spermidine transporter if spermidine is present in high concentrations in the external environment.

Biofilm assays confirmed and extended the previous observations that NspS can negatively regulate biofilm formation by sensing the presence of spermidine in the external environment. NspS was initially identified as a positive regulator of biofilm formation as deletion of the *nspS* gene leads to defects in biofilm formation [16]. Moreover, norspermidine, an analog of spermidine, was shown to increase biofilm formation in the presence of NspS, confirming the role of NspS as a positive regulator. Thus, NspS has a dual role as a positive or negative regulator of biofilm formation depending on the type of polyamines present in the environment. Also, we show that NspS and PotD2 do not function as spermidine transporters based on analyses of polyamine content in Δ*nspS* and Δ*nspSΔpotD1ΔpotD2* mutants. Analysis of the triple mutant lacking all PotD1 paralogs showed no spermidine effect when *nspS* and *potD2* were absent, which reiterates the importance of NspS and spermidine signaling as a mechanism to regulate biofilm formation.

Another piece of information that can be elucidated from the data is the fact that PotD2 showed an effect on biofilm formation, an effect that has not been characterized before this study. In the Δ*nspSΔpotD1ΔpotD2* strain mutant, the biofilm phenotype is decreased when

compared to the biofilm phenotype exhibited by the Δ*nspSΔpotD1* double mutant, indicating that PotD2 has a positive effect on biofilm formation when *nspS* and *potD1* are absent. Furthermore, the biofilm cell densities of Δ*nspSΔpotD1* mutant compared to those of the *ΔnspS* and *ΔpotD1* implicates that neither have a dominant effect on biofilm formation. Our results indicate that spermidine sensing and transport pathways regulate *V. cholerae* biofilms independently of each other. PotD1 and NspS have distinct inputs into the regulation of biofilm formation based on the characterization of the possible epistatic relationship between the two proteins.

In conclusion, previous observations and data obtained from this study allowed for the construction of our working model depicting the mechanisms through which polyamines and polyamine related pathways regulate biofilm formation (Fig. 7). PotD1 functions to negatively regulate biofilm formation. Spermidine imported into the cell via PotD1 has a negative effect on biofilm formation via an unknown mechanism. NspS interacts with norspermidine to have a positive effect on biofilm formation. Conversely, when NspS acts a spermidine signal sensor, a negative effect on biofilm formation is observed. PotD2 is shown to have a positive effect on biofilm formation via interactions with an unknown polyamine. The detailed molecular mechanisms of these effects are under investigation.

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Legends to Figures:

Figure 1. Major Polyamines found in *Vibrio cholerae* (A) and polyamine composition of wild-type *Vibrio cholerae* (B). Diaminopropane, putrescine, cadaverine, and norspermidine are endogenously produced in *V. cholerae*. Spermidine is imported into *V. cholerae* from the LB growth medium (concentration of spermidine in LB media is approximately 40 μM) via transport mechanisms. Diaminooctane is used as an internal standard in polyamine content experiments (as described in the Materials and Methods) (A). Polyamines were extracted, derivatized by benzoylation, and analyzed by HPLC as described in the Materials and Methods. Only data obtained between 5-25 minutes of a 30 minute run is plotted for clarity. Peaks labeled in the wild-type chromatogram respond to putrescine (PUT), diaminopropane (DAP), cadaverine (CAD), norspermidine (NSPD), spermidine (SPD), and diaminooctane (DAO). AU_{254}, absorbance units at 254 nm (B).

Figure 2. **Polyamine composition of *Vibrio cholerae ΔnspS, ΔpotD1,* Δ*nspSΔpotD1,* Δ*nspSΔpotD1ΔpotD2* mutants (A-D).** Polyamines were extracted, derivatized by benzoylation, and analyzed by HPLC as described in the Materials and Methods. Only data obtained between 5-25 minutes of a 30 minute run is plotted for clarity. AU_{254}, absorbance units at 254 nm.

Figure 3. Polyamine composition of wild-type *Vibrio cholerae* (A) and the *ΔnspS, ΔpotD1,* Δ*nspSΔpotD1,* Δ*nspSΔpotD1ΔpotD2* mutants (B-E) in the presence of exogenous spermidine. Polyamines were extracted, derivatized by benzoylation, and analyzed by

HPLC as described in the Materials and Methods. Only data obtained between 5-25 minutes of a 30 minute run is plotted for clarity. 1mM spermidine was added to the growth medium. Peaks labeled in the wild-type chromatogram respond to putrescine (PUT), diaminopropane (DAP), cadaverine (CAD), norspermidine (NSPD), spermidine (SPD), and diaminooctane (DAO). AU_{254}, absorbance units at 254 nm.

Figure 4. Polyamine composition of *Vibrio cholerae* in the presence of 1 mM spermidine- modified protocol. Spermidine was added to the cells at the end of the incubation period. In A-C, cells were incubated for 20 minutes at 4°C. In F-G, cells were immediately processed. Wild-type (A and D), *ΔnspS*, (B and E), *ΔpotD1* (C and F),. Polyamine concentrations were calculated using data collected from the HPLC and were normalized using the internal standard, diaminooctane (DAO).

Figure 5. Multiple sequence analysis of VCA1113 with PotD1 and NspS. Sequences were aligned using ClustalW multiple sequence alignment algorithm of Biology Workbench and colored using the TEXT-SHADE tool (http://workbench.sdsc.edu/).

Figure 6. Biofilm formation in wild-type *Vibrio cholerae* and the *ΔnspS, ΔpotD1, ΔnspSΔpotD1, ΔnspSΔpotD1ΔpotD2* mutants in the presence and absence of exogenous spermidine. Wild-type *V. cholerae* (WT) *ΔnspS, ΔpotD1, ΔnspSΔpotD1,* and *ΔnspSΔpotD1ΔpotD2* cultures were grown in borosilicate tubes in LB media supplemented with 1mM spermidine (light gray bars) or without spermidine (dark gray bars), incubated for 24 hours at 27°C, and quantified as described in the Materials and Methods. Error bars are used to show standard deviations of the three replicate experiments. T-tests were performed to determine statistical significance in biofilm formation upon addition of 1mM spermidine in each strain. Asterisks indicate statistical significance based on the stated P-value.

Figure 7. Model depicting the effects of polyamines and PotD1, NspS, and PotD2 proteins on biofilm formation in *V. cholerae.* Spermidine can affect biofilm formation as an external signal working through the NspS/MbaA signaling pathway. PotD1 functions to negatively regulate biofilm formation. Spermidine imported into the cell via PotD1 has a negative effect on biofilm formation via an unknown mechanism. NspS interacts with norspermidine to have a positive effect on biofilm formation. Conversely, when NspS acts a spermidine signal sensor, a negative effect on biofilm formation is observed. PotD2 is shown to have a positive effect on biofilm formation via interactions with an unknown polyamine.

Table 1
Bacterial strains and plasmids used in this study.

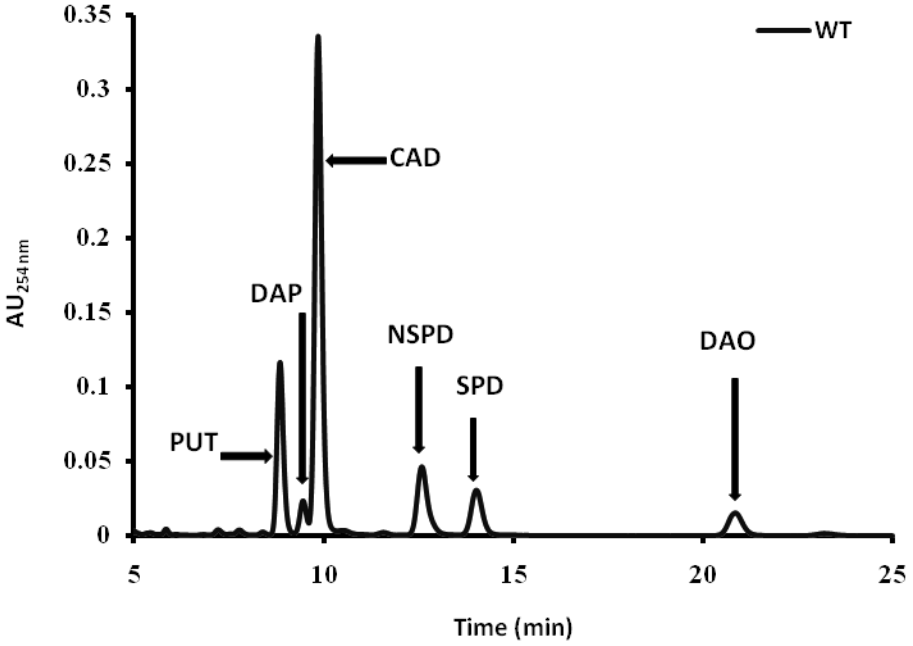
Strains or plasmids	Genotype	Reference or Source
Strains		
E. coli DH5αλpir	supE44 ΔlacU169 hsdR17, recA1 endA1 gyrA96 thi-1 relA1, λpir	[10]
E. coli SM10λpir	*thi thr leu tonA lacY supE recA*::*RP4-2-Tc*::*MuλpirR6K; Km*^{r}	[26] [23]
E. coli AK058	SM10λpir with pMM9 (pWM91::ΔpotD1), Sm^R	[23]
E. coli Ak082	SM10λpir with pMM12 (pWM91:: ΔpotD2ΔpotD1), Sm^R	
V. cholerae PW357	MO10*lacZ*::*vpsLp*→*lacZ*, Sm^R	[11]
V. cholerae PW514	MO10*lacZ*::*vpsLp*→*lacZ*, Δ*nspS*, Sm^R	[16]
V. cholerae AK059	MO10Δ*potD1*, Sm^R	[23]
V. cholerae AK149	MO10 Δ*nspS*Δ*potD1*, Sm^R	This Study
V. cholerae AK150	MO10 Δ*nspS*Δ*pot2*Δ*potD1*, Sm^R	This Study

Cooper, RE. Figure 1

A

Diaminopropane	$H_2N(CH_2)_3NH_2$
Putrescine	$H_2N(CH_2)_4NH_2$
Cadaverine	$H_2N(CH_2)_5NH_2$
Norspermidine	$H_2N(CH_2)_3NH(CH_2)_3NH_2$
Spermidine	$H_2N(CH_2)_4NH(CH_2)_3NH_2$
Diaminooctane	$H_2N(CH_2)_8NH_2$

B



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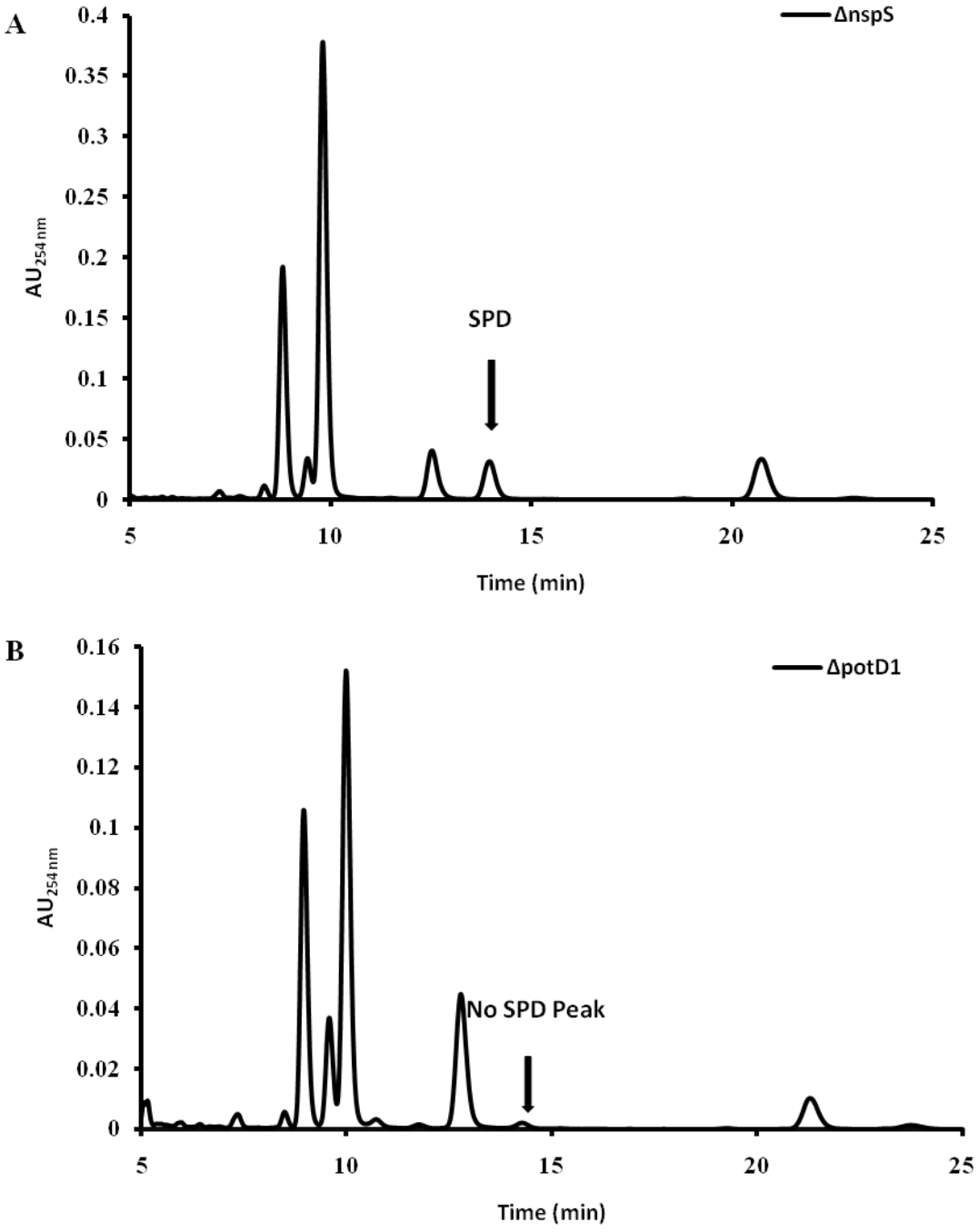

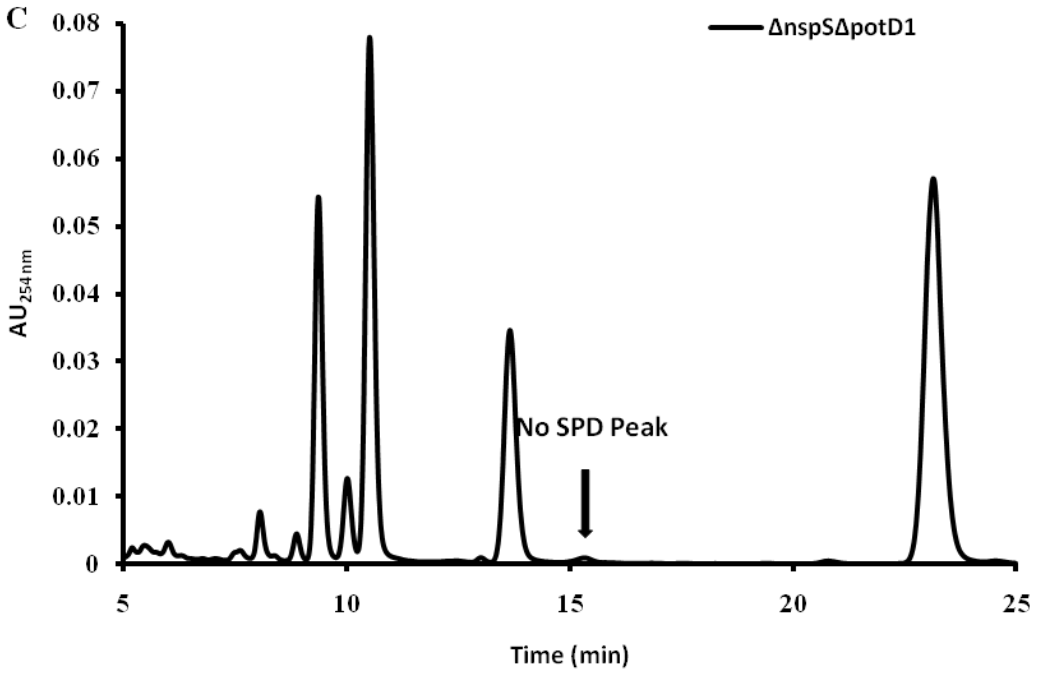
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0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
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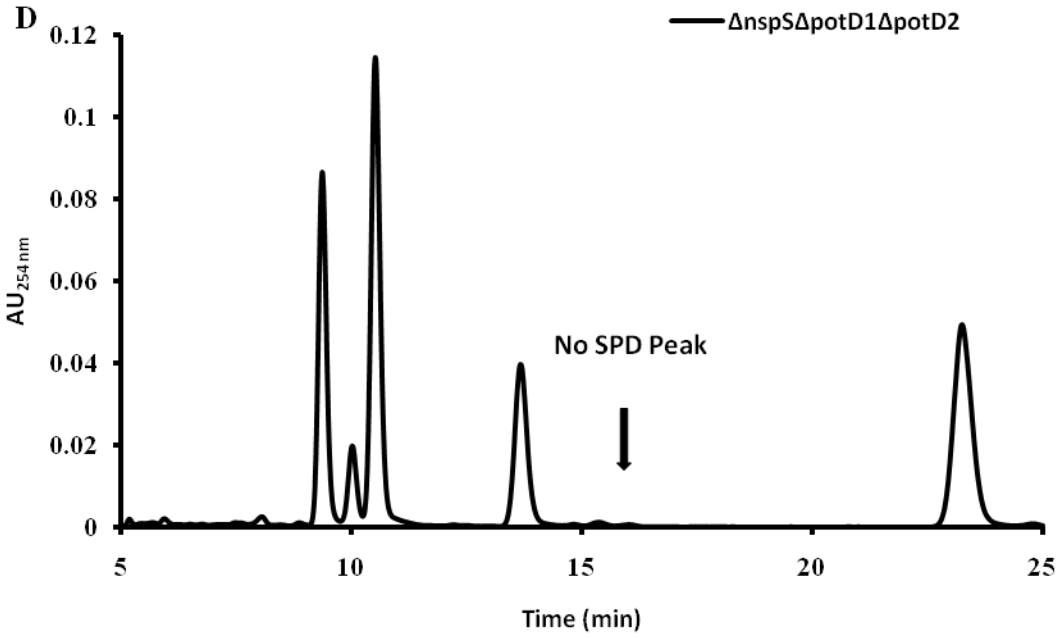
No SPD Peak
5
10
15
20
25
Time (min)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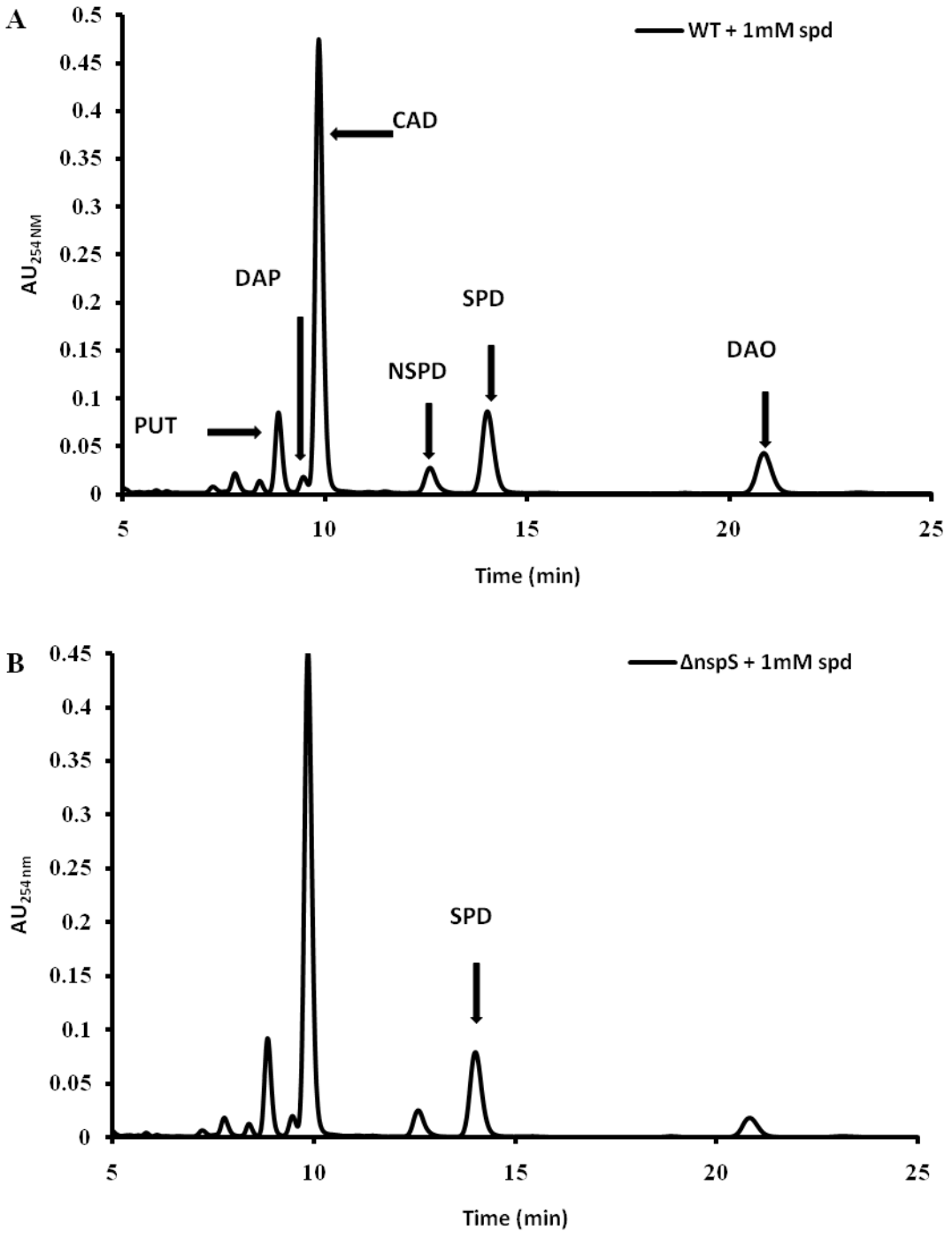
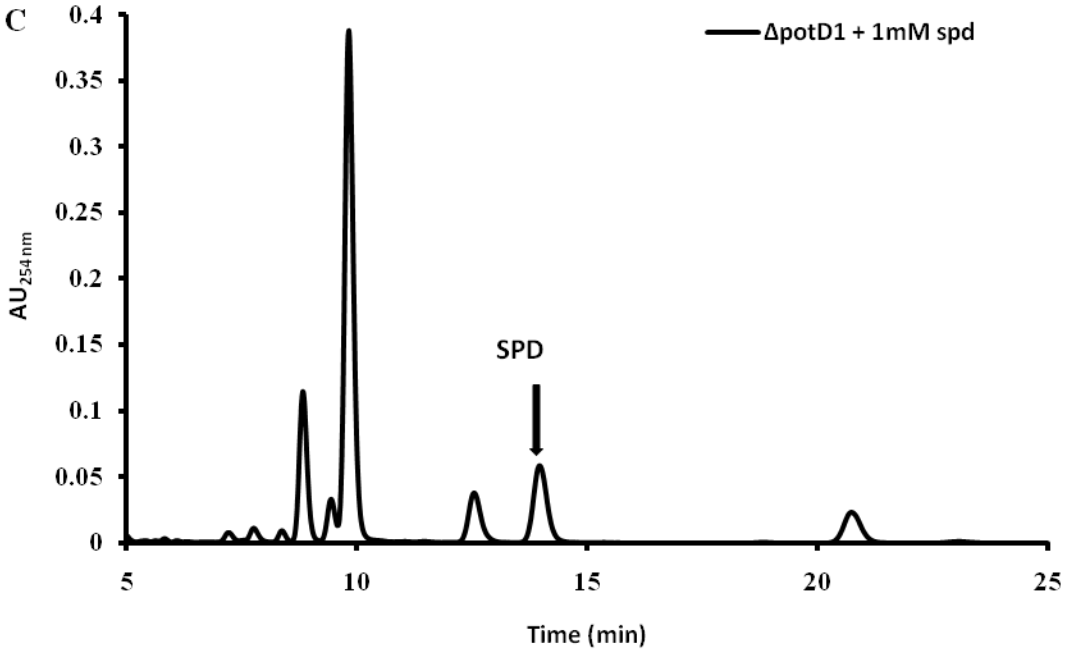
0.02
0
AU254 nm
No SPD Peak
5
10
15
20
25
Time (min)

Cooper, RE. Figure 3

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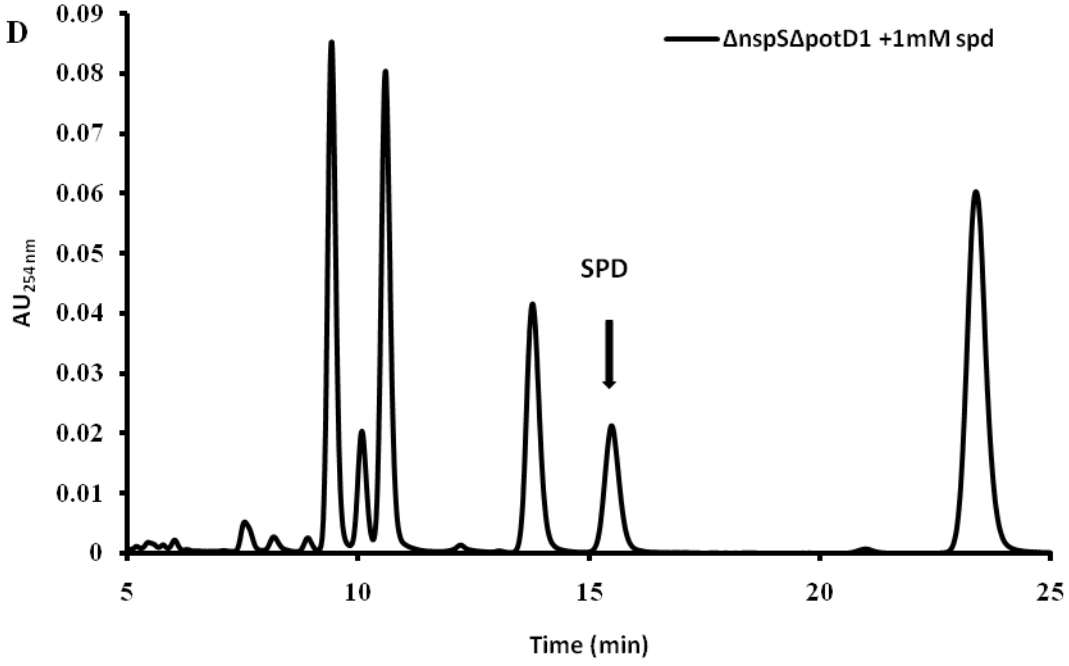
AU254 nm
SPD
Time (min)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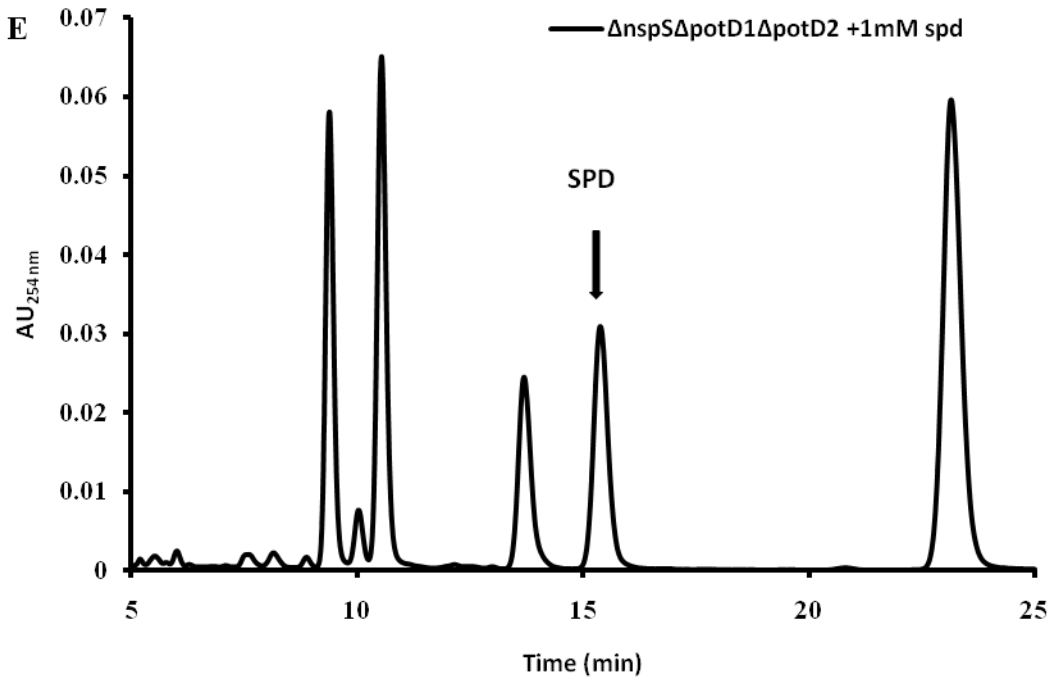
AU254 nm
SPD
Time (min)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0.06
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0.02
0.01
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5
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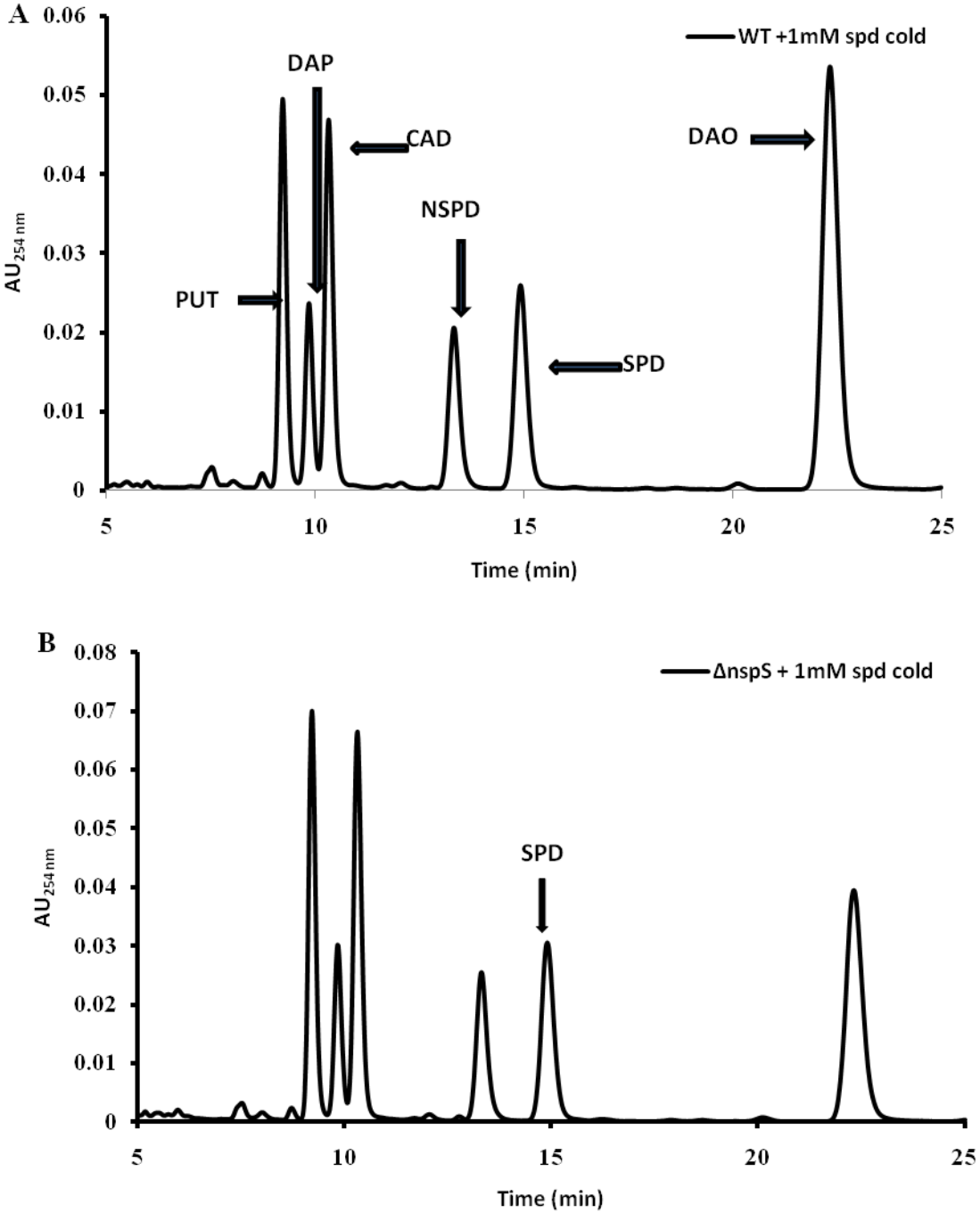
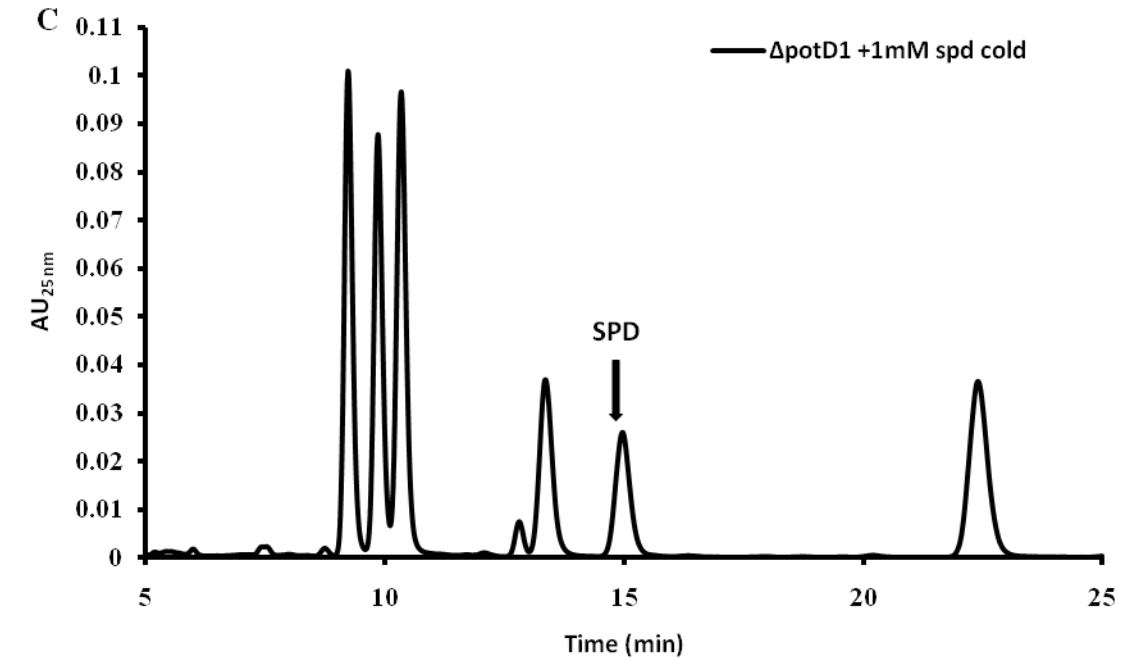
25
Time (min)

Cooper, RE. Figure 4

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SPD
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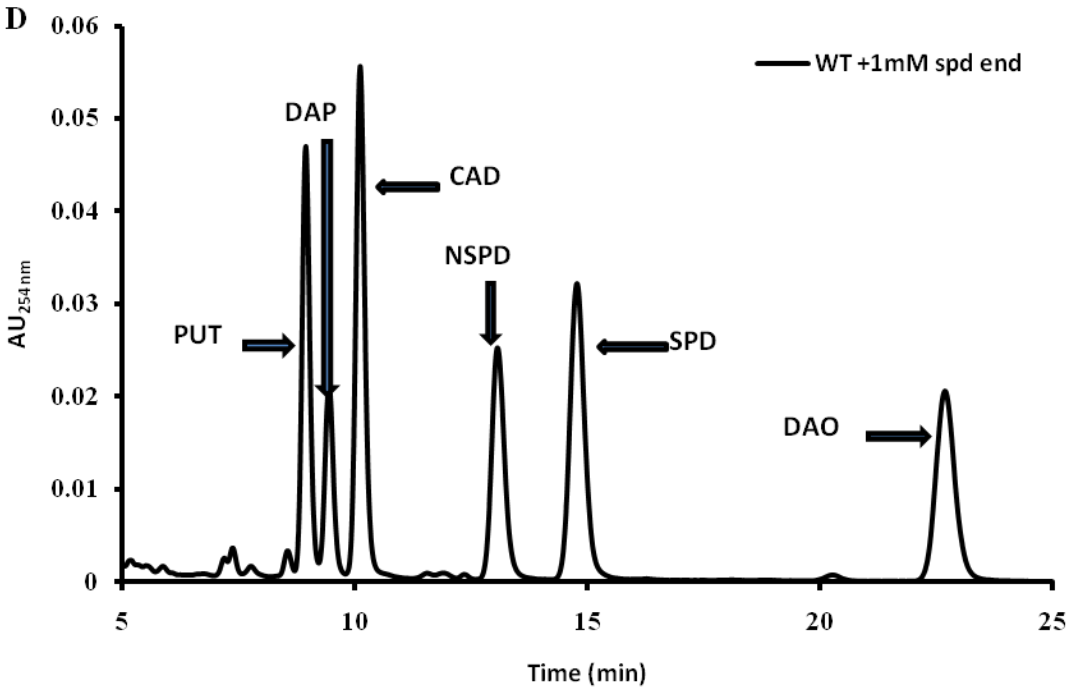
20
25
Time (min)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AU254nm
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0.05
0.04
0.03
0.02
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0
DAP
CAD
NSPD
PUT
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DAO
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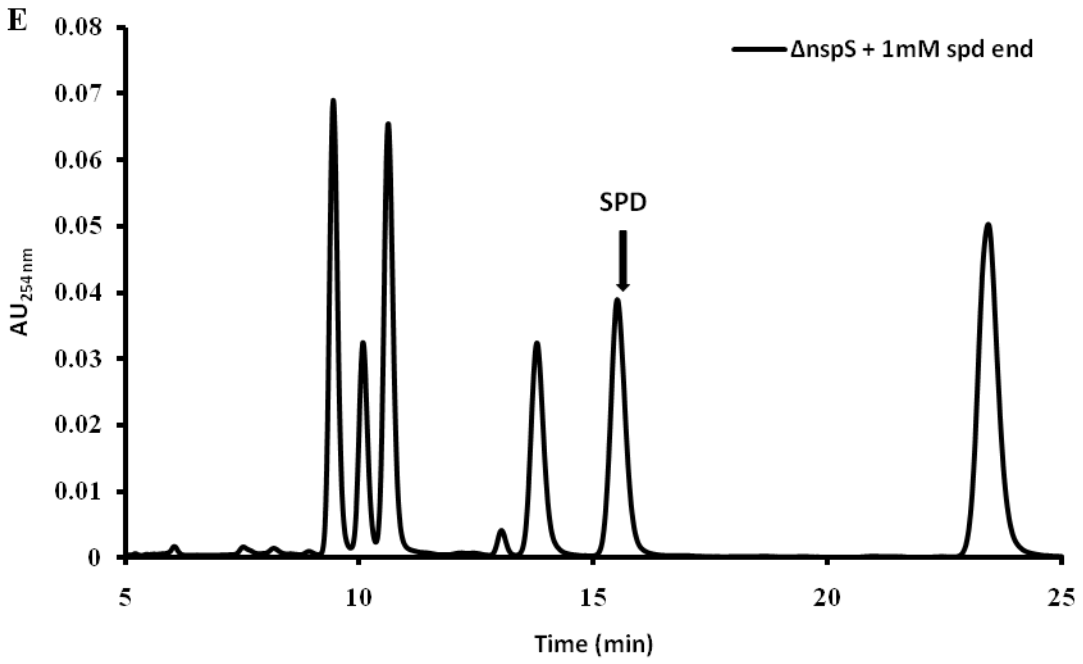
Time (min)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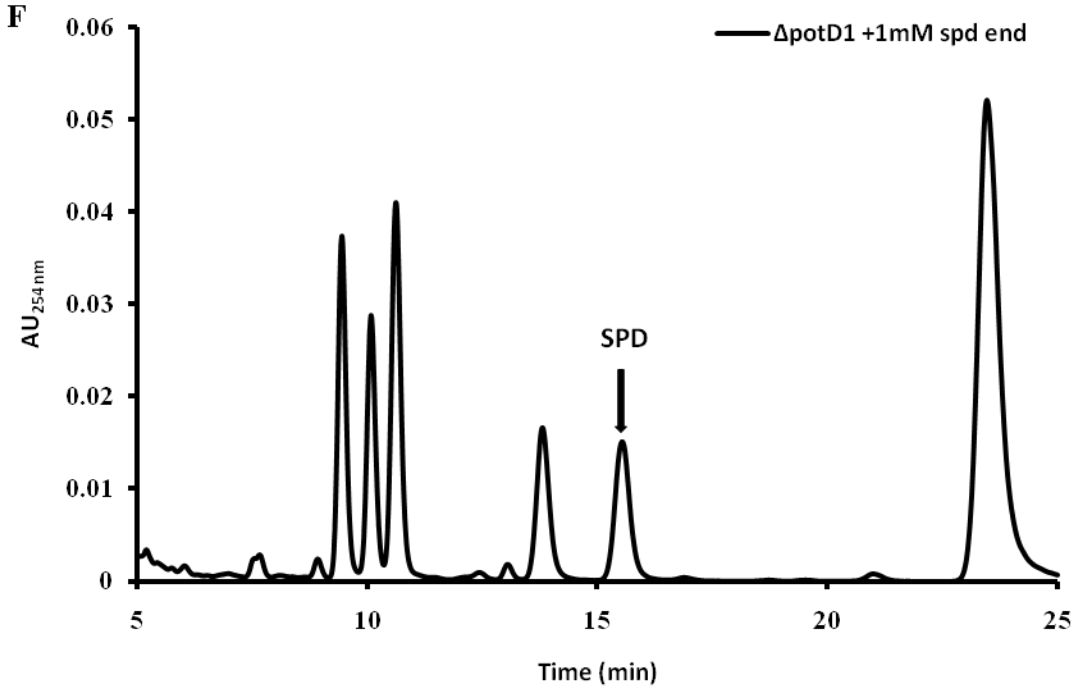
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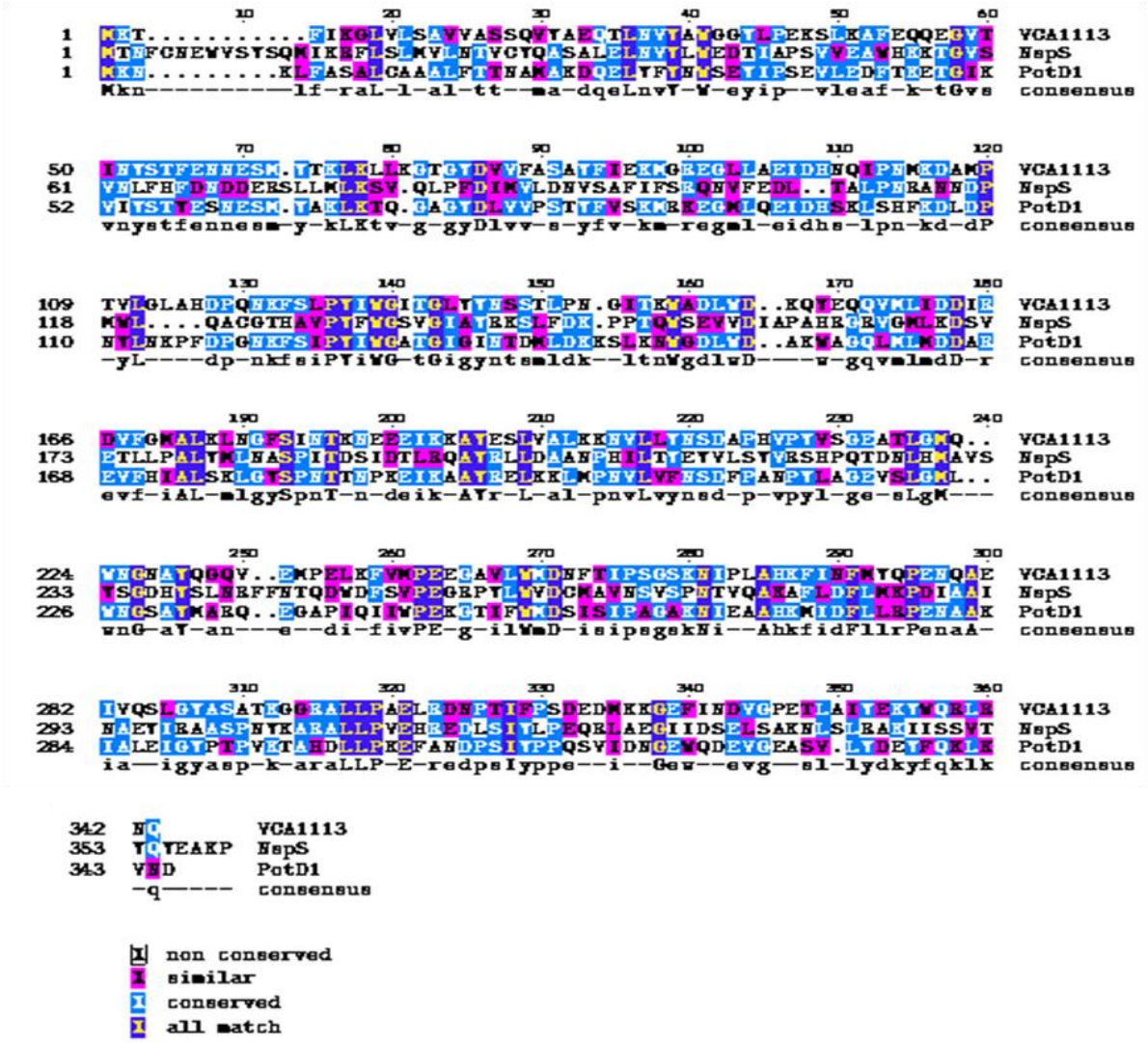
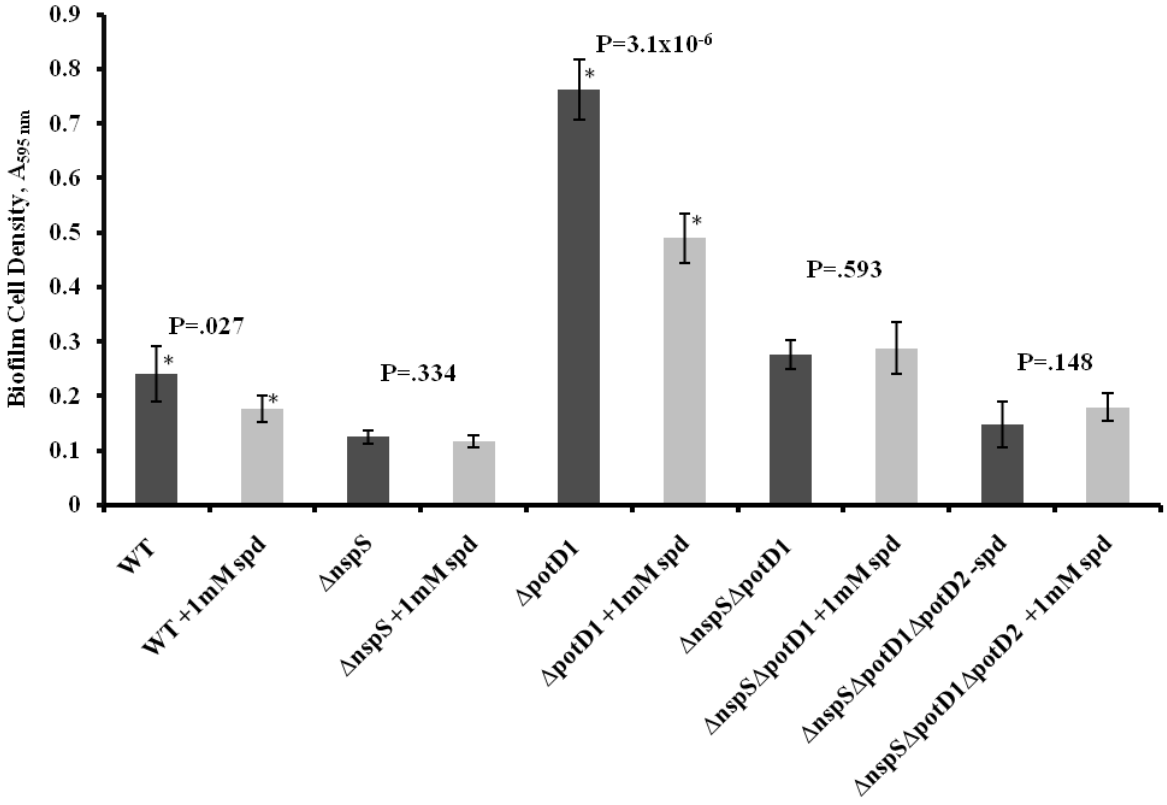
Time (min)

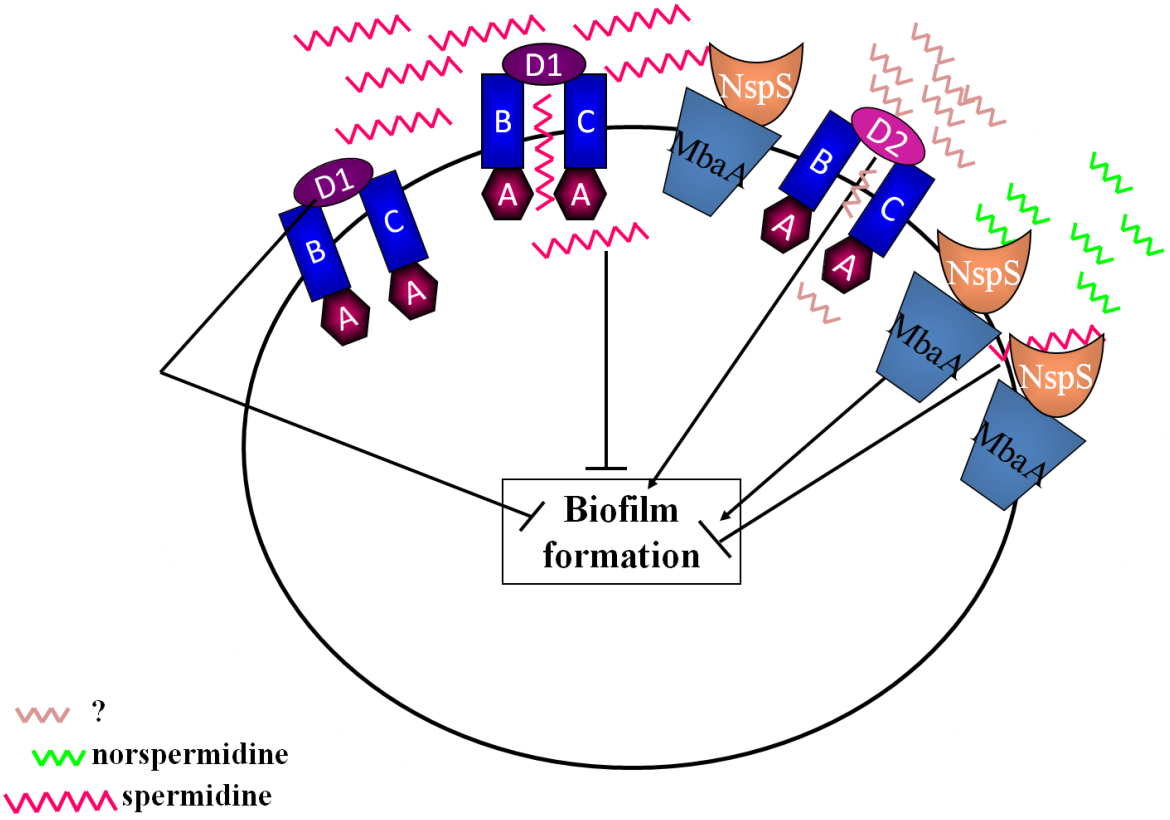
Cooper, RE. Figure 5



Cooper, RE. Figure 6



Cooper, RE. Figure 7



BIOGRAPHICAL SKETCH

Rebecca Elizabeth Cooper was born in Longview, Texas, on July 6, 1986. Becca is the daughter of M. Lynn Cooper of Savannah, Georgia. She attended elementary, middle, and high schools in Savannah, Georgia and graduated as salutatorian from St. Andrew's School in May 2004. In the fall of 2004, she entered Georgia Institute of Technology to study Biology. In May 2008, she was awarded the Bachelor of Science degree in Biology. In the fall of 2008, she accepted a graduate assistantship in the Department of Biology at Appalachian State University and began work toward a Master of Science degree, with a concentration in Cell and Molecular Biology. After receiving her M.S. in Biology from Appalachian State University in August of 2010, Becca will commence work towards her Ph.D. in Biology at Georgia Institute of Technology.